

## Glia relay differentiation cues to coordinate neuronal development in *Drosophila*

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### Abstract

Neuronal birth and specification must be coordinated across the developing brain to generate the neurons that constitute neural circuits. We used the *Drosophila* visual system to investigate how development is coordinated to establish retinotopy, a feature of all visual systems. Photoreceptors achieve retinotopy by inducing their target field in the optic lobe, the lamina neurons, with a secreted differentiation cue (Epidermal Growth Factor; EGF). We find that communication between photoreceptors and lamina cells requires a signaling relay through glia. In response to photoreceptor-EGF, glia produce Insulin-like peptides, which induce lamina neuronal differentiation. Our study identifies a role for glia in coordinating neuronal development across distinct brain regions. Thus reconciling both the timing of column assembly with that of delayed differentiation, as well as the spatio-temporal pattern of lamina neuron differentiation.

**Summary sentence:** Glia coordinate visual system development by responding to Epidermal Growth Factor from photoreceptors to induce target neuron differentiation through local Insulin/Insulin-like Growth Factor signaling.

### Introduction

A key challenge during neural development is to coordinate the birth and specification of diverse neuronal and glial cell-types across different brain regions. To probe this process, we focused on the visual system of *Drosophila*. Like vertebrate visual systems, the fly visual system is organized retinotopically into repeated modular circuits that process sensory input from the entire visual field (1). The lamina is the first ganglion in the optic lobe to receive input from photoreceptors (1). For each of the 800 unit eyes (ommatidia) in the retina, there is a corresponding lamina unit (cartridge) in the optic lobe, made up of 5 lamina neuronal types and multiple glial subtypes. Populating these circuits with the correct number of cells and cell-types, and organizing them spatially, requires that photoreceptor, lamina neuronal and glial development be precisely coordinated.

Photoreceptors develop progressively as a wave of differentiation sweeps across the developing eye imaginal disc, from posterior to anterior (Fig. 1A)(2). New-born photoreceptors promote wave propagation by expressing Hedgehog (Hh), and recruit additional photoreceptor subtypes to developing ommatidia by expressing the Epidermal Growth Factor (EGF), Spitz (Spi)(2). As photoreceptors axons grow into the optic lobes, they are ensheathed by a population of glia, called wrapping glia. Wrapping glial morphogenesis and photoreceptor axon ensheathment occurs in response to Fibroblast Growth Factor (FGF) from photoreceptors (3). Upon arrival in the optic lobes, photoreceptors redeploy Hh and Spi to induce their first target field, the lamina (4–7). Thus, the differentiation wave in the eye disc ultimately drives the development of photoreceptors, wrapping glia and the photoreceptor target-field (lamina neurons).

In the optic lobes, Hh from photoreceptor axons promotes terminal divisions of neuroepithelial cells into equipotent post-mitotic lamina precursors, which express Dachshund (Dac; Fig. 1A,B)(4, 7). These precursors assemble into columns of six to seven cells along photoreceptor axons, also in a Hh-dependent manner (Fig. 1A)(4, 7–9). Hh signaling promotes EGF Receptor (EGFR) expression in

precursors and, according to the current model, makes them competent to respond to Spi, which photoreceptor axons also deliver (Fig. 1A)(5, 6). EGF from photoreceptors drives precursor differentiation into the 5 lamina neuronal types, L1-L5 in each column (marked by Embryonic lethal abnormal vision (Elav), a pan neuronal marker)(Fig 1A,B)(5). Although photoreceptors concomitantly express Hh, which controls precursor cell divisions and column assembly, and EGF, which controls differentiation, precursors differentiate only after column assembly is completed (2, 4, 5). Lamina precursors in each column differentiate according to an invariant spatio-temporal pattern, despite an apparently homogenous differentiation signal from photoreceptors (EGF). In each assembled column of 7 lamina precursors, the most proximal (bottom) and most distal (top) cells differentiate first into L5 and L2, respectively; differentiation then proceeds in a distal-to-proximal (top-to-bottom) sequence, L3 forming next followed by L1 then L4. The two ‘excess’ cells are later cleared by apoptosis (Fig. 1A)(5).

We explored the possibility that other cell types, such as glia, may be involved in coordinating lamina neuronal differentiation with photoreceptors. We found that EGFR in lamina precursors is dispensable for their differentiation into neurons. Instead, photoreceptors signal to wrapping glia with EGF and, in response, wrapping glia induce L1-L4 neuronal differentiation by secreting insulin-like peptides. This intercellular signaling relay couples neuronal differentiation in the lamina with the timing of wrapping glial morphogenesis. We suggest that it accounts for the spatio-temporal pattern of differentiation, which is linked to fate-specification of lamina neurons. Moreover, since glial processes arrive in the lamina after photoreceptors, they may relay the differentiation signal to the lamina with a lag relative to the photoreceptor-delivered signal for column assembly. In this way, glia help reconcile both the timing of column assembly with that of delayed differentiation, as well as the spatio-temporal pattern of lamina neuron differentiation. Glia thus coordinate neuronal development across different ganglia.

### **Glial morphogenesis instructs lamina neuron differentiation**

To explore the coordination of glial morphogenesis with lamina and photoreceptor development, we marked wrapping glia and their processes by using a wrapping glia-specific driver to express membrane-targeted GFP (Fig. 1B, Movie S1)(10). Wrapping glia are basal to photoreceptor cell bodies in the eye disc (3, 11–13). Their processes wrap photoreceptor axons through the optic stalk and in the developing lamina (Fig. 1B)(12). Wrapping glial extension along photoreceptors into the optic lobes was progressive, such that their processes invaded the lamina further in older columns, progressing as did differentiation (Fig. 1B). Therefore, the leading edge of wrapping glial processes arriving in the optic lobes correlated with the front of neuronal differentiation in the lamina (Fig. 1B). When we disrupted wrapping glial morphogenesis and extension into the optic lobes by expressing a dominant negative form of the FGF Receptor, Heartless ( $Htl^{DN}$ )(3) in wrapping glia, we observed that the triangular front of differentiation indicative of sequential L1-L4 differentiation was disrupted (Fig. 1C). Differentiating lamina neurons only occupied the distal (top)-most positions in columns. Presumptive L5 neurons were still present but differentiated with a delay of ~3 columns (Figs. 1C). This suggests that wrapping glia are involved in lamina differentiation.

### **EGFR is dispensable in lamina precursors for differentiation**

Since both wrapping glial morphogenesis and EGF from photoreceptors are required for lamina neuron differentiation (5), we revisited the original model to further characterize the role of EGF from photoreceptors. Rhomboid (Rho) proteins cleave Spi, making it active for secretion (6, 14). Rho3 is specifically localized to photoreceptor axons and *rho3* mutants lack photoreceptor axon-derived Spi, and lamina precursors fail to differentiate although they are recruited into columns (6). In these mutants, Rho1 maintains normal Spi secretion from the cell body such that photoreceptors are specified normally and project appropriately to the lamina (6). Rescue experiments have demonstrated that the *rho3* mutant phenotype can be entirely attributed to loss of Spi secretion from photoreceptor axons (6). As expected, differentiating lamina neurons exhibited dually phosphorylated Mitogen-Activated Protein Kinase (dpMAPK), a readout for Receptor Tyrosine Kinase (RTK) activity, which was lost in *rho3* mutants. L1-L4s were eliminated in *rho3* mutants as previously reported; however, L5s differentiated but with a delay,



indicating that they follow a distinct differentiation program from L1-L4 and do not require EGF from photoreceptor axons (Fig. S1C-G). We thereafter focused only on L1-L4 differentiation, which is abolished in the absence of EGF from photoreceptor axons.

Although it is unambiguous that EGF from photoreceptors is required for lamina neuronal differentiation, the lack of cell type-specific tools at the time the original model was formulated precluded testing whether EGFR and MAPK are required specifically in the lamina for L1-L4 to differentiate (5). To test this, we used a lamina-specific Gal4 (*l0*) to drive a dominant negative form of EGFR (EGFR<sup>DN</sup>) in lamina precursors and in differentiating lamina neurons (Fig. S2A). This did not prevent lamina neuron differentiation as Elav-positive cells were observed. However, lamina morphology was disrupted, likely due to apoptosis of lamina cells (Fig. S2A). Preventing cell death by expressing the baculovirus caspase inhibitor P35 along with EGFR<sup>DN</sup>, restored lamina morphology, revealing that the pattern of differentiation was unaffected by blocking EGFR activity in the lamina (Fig. 1E,H). Thus, although EGFR appears to be required in lamina cells for their survival, they do not require it to differentiate. Thus implying that photoreceptors do not communicate directly with lamina precursors through EGF.

Although EGFR is not required for differentiating lamina neurons, MAPK signaling was active in these cells (Fig. S1A), likely downstream of another RTK (*l5*). To test whether MAPK signaling is required for lamina neuron differentiation, we blocked transcription downstream of MAPK by expressing an activated form of the negative regulator of the pathway, Anterior open (*Aop*<sup>ACT</sup>), in lamina precursors (Fig. S2B,C). Differentiation was blocked and lamina morphology was also disrupted (again, likely due to apoptosis)(Fig. S2C). Blocking cell death by co-expressing P35 with *Aop*<sup>ACT</sup> restored lamina morphology. However, lamina neuron differentiation was still prevented (Figs. 1F,H). To test whether MAPK activation could drive ectopic neuronal differentiation we expressed an activated form of MAPK (MAPK<sup>ACT</sup>) in the lamina (Fig. 1G). Instead of a triangular front of differentiation, indicative of sequential differentiation, most lamina columns differentiated immediately after formation and many more differentiated cells were present (Fig. 1G,H). Importantly, MAPK was sufficient to drive lamina differentiation even in the absence of EGF from photoreceptors in a *rho3* mutant (Supplementary text and Fig. S2). These data show that MAPK signaling in lamina precursor cells is both necessary and sufficient for lamina neuronal differentiation.

### **Lamina differentiation requires photoreceptor-activated EGFR in glia**

Since photoreceptors signal through EGF but lamina precursors do not respond to it, we hypothesized that photoreceptors signal to wrapping glia, which relay cues to lamina precursors. We tested whether glia respond to Spi from photoreceptor axons: Wrapping glial nuclei (located in eye discs) had reduced levels of dpMAPK in *rho3* mutants relative to controls (Fig. 2A-C; remaining activity likely due to FGFR signaling), indicating that photoreceptor axon-derived Spi activated the EGFR pathway in wrapping glia.

To evaluate the function of active EGFR signaling in wrapping glia we used a wrapping glia-specific Gal4 line to express EGFR<sup>DN</sup>. Glial ensheathment of photoreceptor axons was not affected by this manipulation (Movies S1-3). However, the L1-L4 triangular front of neuronal differentiation was absent (Fig. 2D,E, J). L5 differentiation was unaffected (Figs. 2D,E, J), as L5-specific markers, were expressed in the proximal row of the developing lamina, (Fig. S2H; See Table S1 for description of neuronal subtype-specific markers; (*l6*)). These data show that active EGFR signaling in wrapping glia is necessary for L1-L4 but not L5 differentiation.

Together our data suggest that photoreceptors do not signal directly to lamina precursors. Rather, wrapping glia respond to EGF from photoreceptors to induce L1-L4 differentiation. We therefore asked whether activating the EGFR pathway in wrapping glia alone could bypass the requirement for EGF from photoreceptors and rescue L1-L4 differentiation in the lamina. We expressed an activated form of EGFR (EGFR<sup>ACT</sup>) in wrapping glia in a *rho3* mutant background. In this genotype, photoreceptor axons could not secrete EGF but EGFR signaling was activated only in wrapping glia. Lamina differentiation was rescued and all L1-L4 cell types were recovered (Figs. 2G,I,J, S4 and Table S1). Similar results were obtained when we expressed activated Ras (Ras<sup>V12</sup>) in wrapping glia in *rho3* mutants (Fig. S2I,J). These

results argue that EGF from photoreceptor axons activates EGFR in wrapping glia, which is both necessary and sufficient to induce L1-L4 differentiation.

### **Glial Insulin-like peptides induce lamina differentiation**

Since lamina precursors require active MAPK signaling to differentiate into neurons (Fig. 1F-H), we reasoned that the differentiation signal from wrapping glia must act through an RTK upstream of MAPK. The *Drosophila* genome encodes 20 RTKs, although only 10 lie upstream of MAPK signaling (15). Of these, we focused on the Insulin Receptor (InR) as, in other instances, glia can use Insulin/Insulin-like Growth Factor signaling to communicate with neural progenitors (17, 18). In *Drosophila* seven Insulin-like peptides (Ilp1-Ilp7) bind to and activate the sole *Drosophila* InR (19). Chico is the only Insulin Receptor Substrate in *Drosophila* (20), and acts to stabilize binding of activated InR to PI3-Kinase (PI3K) and Growth factor receptor-bound protein 2 (Grb2), leading to activation of PI3K and MAPK signaling, respectively (Fig. S3A)(21, 22). Thus, *chico* mutants have low levels of Insulin signaling. Differentiating L1-L4s were missing in large regions of all *chico* mutant laminae (~20% of the lamina; Fig. 3B). In other regions of the same mutant brains, no or partial loss of differentiated lamina neurons was evident. A viable hypomorphic allelic combination for *InR* showed similar differentiation defects (Fig. S3B)(21, 22). To determine whether InR and Chico were signaling through PI3K or through MAPK, we used Chico constructs that rescue both or only one or the other of the downstream signaling pathways (21): InR requires signaling through MAPK but not PI3K for lamina neuronal differentiation (Supplementary text and Fig. S3C-E).

To activate InR in lamina precursors and induce L1-L4 neuronal differentiation, wrapping glia must secrete Ilps in response to EGF from photoreceptors. Although the central brain Insulin Producing Cells secrete several Ilps that act systemically, Ilps can also be developmentally and regionally expressed (17, 18, 23). Ilp2, Ilp3 and Ilp5 are only expressed in Insulin Producing Cells in the central brain complex (23, 24). We were unable to test for wrapping glial expression of Ilp1 and Ilp4 due to a lack of reporters. However, reporter constructs for Ilp6 and Ilp7 both drove GFP expression in wrapping glia (Fig. 3C,D) and Ilp6-Gal4 expression in wrapping glia was dramatically decreased in *rho3* mutants (Fig. S3F,G). Neither *Ilp6* nor *Ilp7* single mutants showed defects in lamina neuronal differentiation (Fig. S3H,I); however, Ilps are known to act redundantly and removal of some Ilps can lead to compensatory regulation by others (24). Therefore, in order to disrupt Ilp function, we ectopically expressed a secreted antagonist of Ilps, Imaginal morphogenesis protein-L2 (Imp-L2)(25–27), in large actin-flip-out clones (Fig. S3J). Consistent with the *chico* and *InR* mutant data, blocking Ilp activity by ImpL2 mis-expression led to an almost complete loss of L1-L4 neuronal differentiation (Fig. S3J). Thus, secreted Ilps are required for lamina differentiation.

Since Ilp6 in wrapping glia is lost in the absence of EGF from photoreceptors, we asked whether restoring Ilp6 in wrapping glia was sufficient to induce L1-L4 differentiation. Expressing Ilp6 in wrapping glia in a *rho3* mutant background rescued the L1-L4 triangular front of differentiation (Fig. 3F,H). Moreover, all L1-L4 subtypes were recovered (Figs. S3K; Fig. S4 and Table S1). dpMAPK expression in the lamina was also restored (Fig. S3L,M), further confirming that Ilp6 is sufficient to activate the MAPK branch of Insulin signaling during lamina neuronal differentiation. We also tested whether ectopically activating InR (with InR<sup>ACT</sup>) in the lamina could bypass all exogenous cues to rescue lamina neuronal differentiation in a *rho3* mutant background (Fig. 3G,H). Although several rows of Elav positive cells were recovered, they were disorganized (Fig. 3G,H). These cells included L1s, L2/3s, and L5s but no L4s (Fig. S3N, Fig. S4 and Table S1). Altogether our data show that wrapping glia receive EGF from photoreceptors and respond to produce Insulin-like peptides that induce differentiation of lamina precursors by activating MAPK.

### **The signaling relay may serve to delay differentiation**

Since photoreceptors could signal directly to lamina precursors (4, 7), but instead act through glia, we sought to understand the advantages of this relay mechanism. Glial processes arrive in the optic lobes after photoreceptor axons (Fig. 1B). Thus, the relay may delay the differentiation cue to ensure that

column assembly is completed before differentiation initiates. To test this, we supplied Ilp6 directly from photoreceptors in *rho3* mutants to bypass glial signaling (Fig. 4). We expressed Ilp6 with two pan-photoreceptor drivers that differed in the onset of their expression: the first was expressed in early-born photoreceptors, and the second was delayed relative to photoreceptor birth (Fig. 4C,D). We predicted that column assembly (6-7 lamina precursors/column) would not be completed reliably when photoreceptors delivered assembly and differentiation cues simultaneously (early driver), as lamina precursors would differentiate too early. However, if the differentiation cue was delayed, the correct number of lamina precursors would assemble into columns before differentiating. While early photoreceptor-delivered Ilp6 expression rescued lamina neuronal differentiation in a *rho3* mutant, fewer lamina precursors incorporated into columns on average ( $4.4 \text{ lamina precursors/column} \pm 0.99 \text{sd}$ ;  $N=4$  optic lobes; Fig. 4C). Moreover, Elav expression initiated in the youngest column that contained 4 or fewer lamina precursors, indicating that they were still being assembled (Fig. 4C). When Ilp6 was expressed with the delayed-onset photoreceptor driver in *rho3* mutants, lamina neuronal differentiation had only initiated in old columns at  $\sim 10 \text{hrs}$  After Puparium Formation (APF) (Fig. 4D). However, columns contained 6-7 precursors each ( $\pm 0.88 \text{sd}$ ;  $N=4$  optic lobes; Fig. 4C), indicating that column assembly was less disrupted compared with early-onset Ilp6 expression. By  $\sim 15 \text{hrs}$  APF, all photoreceptors expressed Ilp6, and neuronal differentiation was widespread, suggesting that differentiation could ‘catch up’ (Fig. 4E). Nonetheless, the pattern was disrupted, as the number of neurons in each column did not reflect the age of the column (Fig. 5E). These data suggest that the relay from photoreceptors to wrapping glia to lamina precursors may function to segregate column assembly from differentiation in time.

## Discussion

In *Drosophila*, photoreceptors establish retinotopy between the retina and their target field, the lamina, by inducing lamina units, each containing 5 neurons. This is a multi-step process requiring lamina precursor generation and assembly into naïve columns, followed by their differentiation into L1-L5 according to an invariant spatio-temporal pattern. We showed that L1-L4 differentiation is the consequence of an intercellular signaling relay from photoreceptors to wrapping glia and then to lamina precursors (Fig. 5). Rather than instructing lamina neuronal differentiation directly, EGF secreted from photoreceptor axons activates the EGFR pathway in glia (Fig. 5); in turn, glia induce lamina precursors to differentiate into L1-L4 through local Insulin and MAPK signaling (Fig. 5). Although photoreceptor axons require InR to target the lamina (28), targeting was unaffected in *rho3* mutants (where L1-L4 do not differentiate due to reduced glial-Ilps). It is therefore unlikely that wrapping glial Ilps also guide photoreceptor axon targeting.

Intercellular signaling relays are used in various contexts during development (17, 18, 29, 30). In the context of the lamina, the glial relay serves several purposes: (i) The delayed arrival of glial processes into the optic lobes relative to photoreceptor axons temporally segregates column assembly from differentiation (Fig. 4). The relay from photoreceptors to glia to lamina precursors could therefore be a mechanism to ensure that column assembly is completed before differentiation initiates, leading to reproducible numbers of precursors in each column (Fig. 4). (ii) The spatiotemporal pattern of lamina neuronal differentiation is likely a consequence of being coupled to progressive glial morphogenesis. Glial wrapping is itself coordinated independently by FGF from photoreceptors (Fig. 5)(3). Thus, photoreceptors independently regulate the ability of wrapping glia to induce differentiation in the lamina as well as the timing and pattern of this induction. All wrapping glia-driven rescues of the *rho3* mutant generated all lamina neuron subtypes (Fig. S4 and Table S1). However, this was not the case for lamina-driven rescues of *rho3*, which produced aberrant subtypes while sometimes lacking others (Fig. S4 and Table S1). By signaling through glia, photoreceptors may be translating a homogenous cue (EGF) into a spatio-temporally graded one, which appears essential for diversifying (L1-L4) neuronal fates. (iii) Glial cells may be well suited for integrating sparse cues to interpret them into stronger or more robust signals (31). Thus, by amplifying cues from photoreceptors, glia may help reduce noise or variability of the signaling outcome.

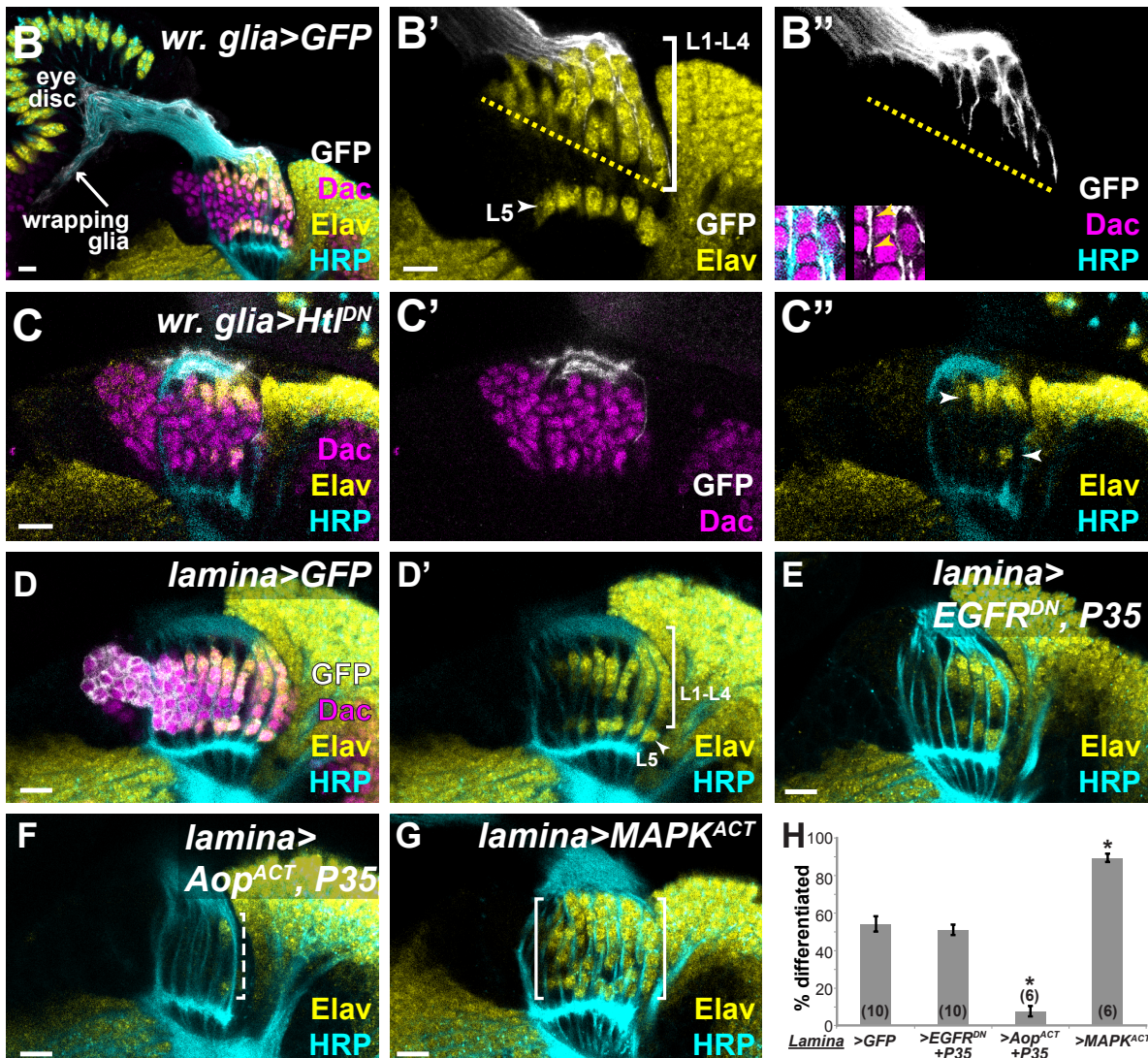
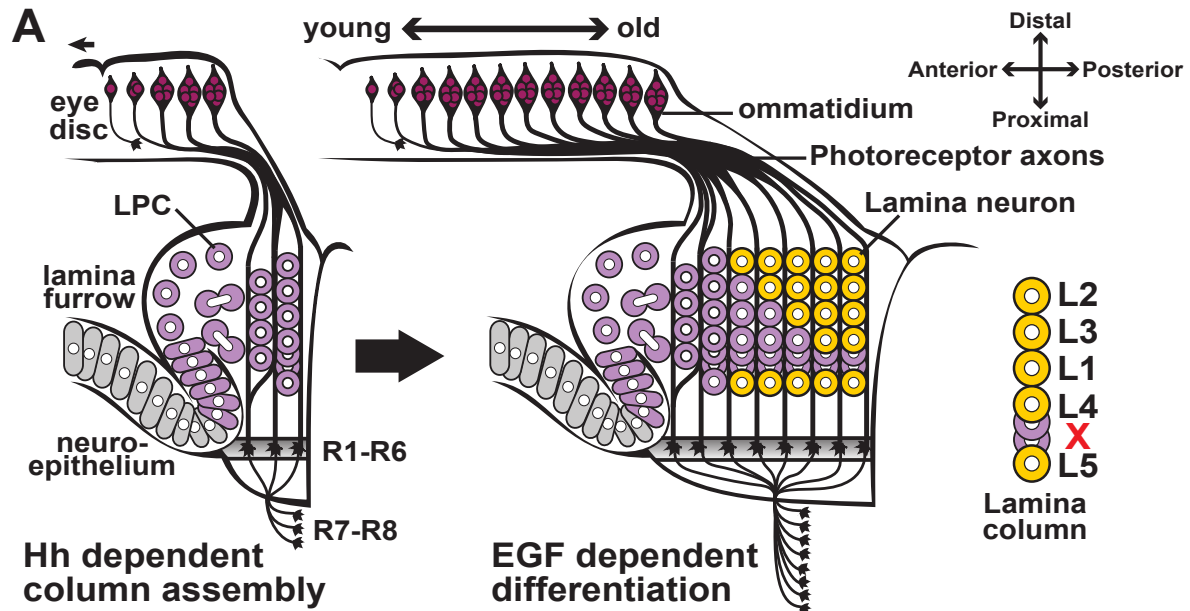
## References

1. D. Hadjiconomou, K. Timofeev, I. Salecker, A step-by-step guide to visual circuit assembly in *Drosophila*. *Curr. Opin. Neurobiol.* **21**, 76–84 (2011).
2. J.-Y. Roignant, J. E. Treisman, Pattern formation in the *Drosophila* eye disc. *Int. J. Dev. Biol.* **53**, 795–804 (2009).
3. S. R. Franzdóttir *et al.*, Switch in FGF signalling initiates glial differentiation in the *Drosophila* eye. *Nature*. **460**, 758–61 (2009).
4. Z. Huang, S. Kunes, Hedgehog, transmitted along retinal axons, triggers neurogenesis in the developing visual centers of the *Drosophila* brain. *Cell*. **86**, 411–422 (1996).
5. Z. Huang, B. Z. Shilo, S. Kunes, A retinal axon fascicle uses spitz, an EGF receptor ligand, to construct a synaptic cartridge in the brain of *Drosophila*. *Cell*. **95**, 693–703 (1998).
6. S. Yogeve, E. D. Schejter, B. Z. Shilo, Polarized secretion of *Drosophila* EGFR ligand from photoreceptor neurons is controlled by ER localization of the ligand-processing machinery. *PLoS Biol.* **8**, e1000505 (2010).
7. Z. Huang *et al.*, Signals transmitted along retinal axons in *Drosophila*: Hedgehog signal reception and the cell circuitry of lamina cartridge assembly. *Development*. **125**, 3753–64 (1998).
8. A. Sugie, D. Umetsu, T. Yasugi, K.-F. Fischbach, T. Tabata, Recognition of pre- and postsynaptic neurons via nephrin/NEPH1 homologs is a basis for the formation of the *Drosophila* retinotopic map. *Development*. **137**, 3303–3313 (2010).
9. D. Umetsu, S. Murakami, M. Sato, T. Tabata, The highly ordered assembly of retinal axons and their synaptic partners is regulated by Hedgehog/Single-minded in the *Drosophila* visual system. *Development*. **133**, 791–800 (2006).
10. B. D. Pfeiffer *et al.*, Tools for neuroanatomy and neurogenetics in *Drosophila*. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 9715–9720 (2008).
11. R. Rangarajan, Q. Gong, U. Gaul, Migration and function of glia in the developing *Drosophila* eye. **3292**, 3285–3292 (1999).
12. T. N. Edwards, A. C. Nuschke, A. Nern, I. A. Meinertzhagen, Organization and metamorphosis of glia in the *Drosophila* visual system. *J. Comp. Neurol.* **520**, 2067–2085 (2012).
13. M. Silies *et al.*, The eye imaginal disc as a model to study the coordination of neuronal and glial development. *Fly (Austin)*. **6934**, 71–70 (2015).
14. S. Urban, J. R. Lee, M. Freeman, A family of rhomboid intramembrane proteases activates all *Drosophila* membrane-tethered EGF ligands. *EMBO J.* **21**, 4277–4286 (2002).
15. R. Sopko, N. Perrimon, Receptor Tyrosine Kinases in *Drosophila* Development. *Cold Spring Harb. Perspect. Biol.* **5** (2013).
16. M. Y. Pecot *et al.*, Sequential axon-derived signals couple target survival and layer specificity in the *Drosophila* visual system. *Neuron*. **82**, 320–333 (2014).
17. J. M. Chell, A. H. Brand, Nutrition-Responsive Glia Control Exit of Neural Stem Cells from Quiescence. *Cell*. **143**, 1161–1173 (2010).
18. R. Sousa-nunes, L. L. Yee, A. P. Gould, Fat cells reactivate quiescent neuroblasts via TOR and glial insulin relays in *Drosophila*. *Nature*. **471**, 508–512 (2011).
19. W. Brogiolo *et al.*, An evolutionarily conserved function of the *Drosophila* insulin receptor and insulin-like peptides in growth control. *Curr. Biol.* **11**, 213–221 (2001).
20. R. Böhni *et al.*, Autonomous control of cell and organ size by CHICO, a *Drosophila* homolog of vertebrate IRS1–4. *Cell*. **97**, 865–875 (1999).
21. C. Slack *et al.*, The Ras-Erk-ETS-Signaling Pathway Is a Drug Target for Longevity. *Cell*. **162**, 72–83 (2015).
22. S. Oldham, E. Hafen, Insulin/IGF and target of rapamycin signaling: A TOR de force in growth control. *Trends Cell Biol.* **13**, 79–85 (2003).
23. D. R. Nässel, J. Vanden Broeck, Insulin/IGF signaling in *Drosophila* and other insects: Factors that regulate production, release and post-release action of the insulin-like peptides. *Cell. Mol. Life Sci.* **73**, 271–290 (2016).

24. S. Grönke, D. Clarke, S. Broughton, T. D. Andrews, L. Partridge, Molecular Evolution and Functional Characterization of Drosophila Insulin-Like Peptides. *PLoS Genet.* **6**, e1000857 (2010).
25. N. Alic, M. P. Hoddinott, G. Vinti, L. Partridge, Lifespan extension by increased expression of the Drosophila homologue of the IGFBP7 tumour suppressor. *Aging Cell.* **10**, 137–147 (2011).
26. N. Arquier *et al.*, Drosophila ALS Regulates Growth and Metabolism through Functional Interaction with Insulin-Like Peptides. *Cell Metab.* **7**, 333–338 (2008).
27. B. Honegger *et al.*, Imp-L2, a putative homolog of vertebrate IGF-binding protein 7, counteracts insulin signaling in Drosophila and is essential for starvation resistance. *J. Biol.* **7**, 10 (2008).
28. J. Song, L. Wu, A. Xhen, R. A. Kohanski, L. Pick, Axons Guided by Insulin Receptor in Drosophila Visual System. *Science* (80-. ). **300**, 502–505 (2003).
29. Z. Ma, T. Stork, D. E. Bergles, M. R. Freeman, Neuromodulators signal through astrocytes to alter neural circuit activity and behaviour. *Nature.* **539**, 428–432 (2016).
30. J. L. Leatherman, S. Dinardo, Germline self-renewal requires cyst stem cells and stat regulates niche adhesion in Drosophila testes. *Nat. Cell Biol.* **12**, 806–811 (2010).
31. D. K. Ma, G. L. Ming, H. Song, Glial influences on neural stem cell development: Cellular niches for adult neurogenesis. *Curr. Opin. Neurobiol.* **15**, 514–520 (2005).

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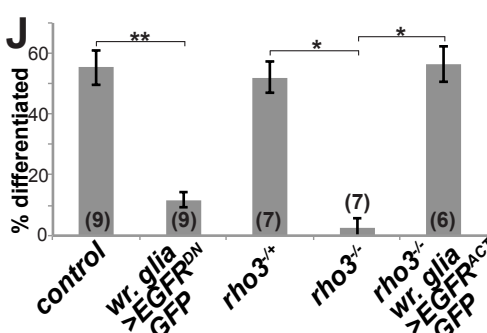
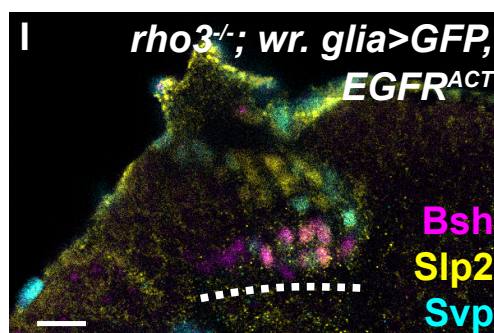
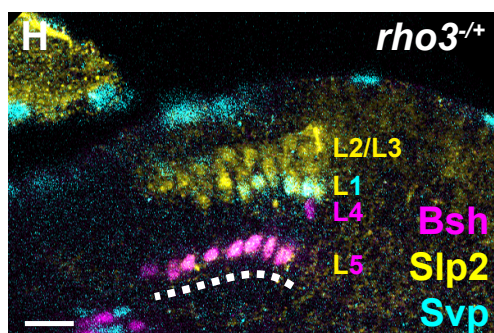
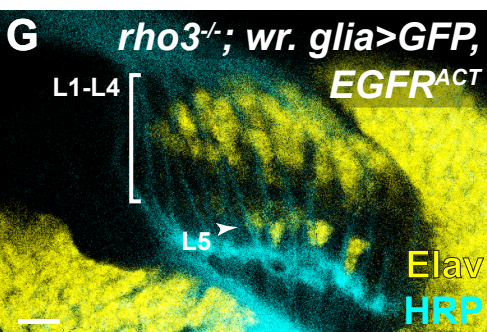
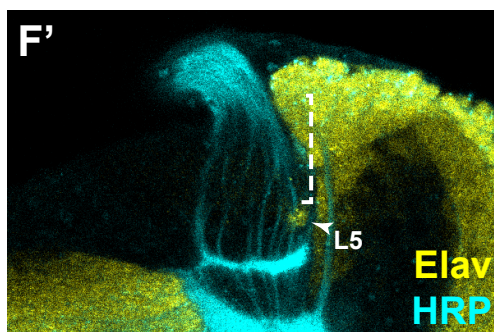
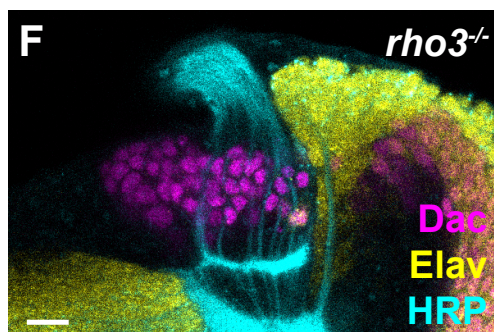
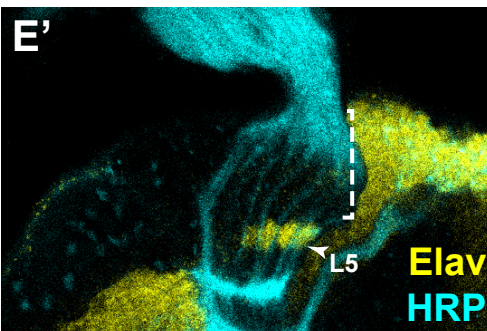
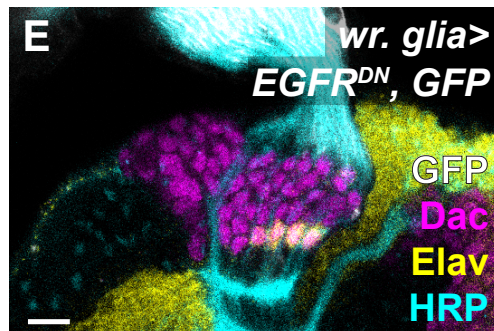
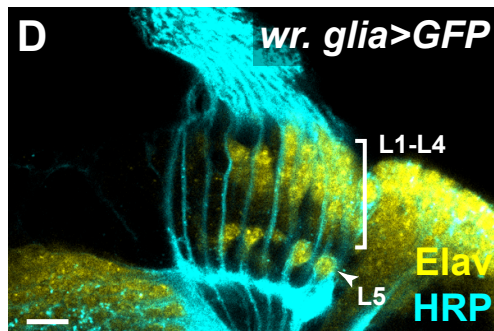
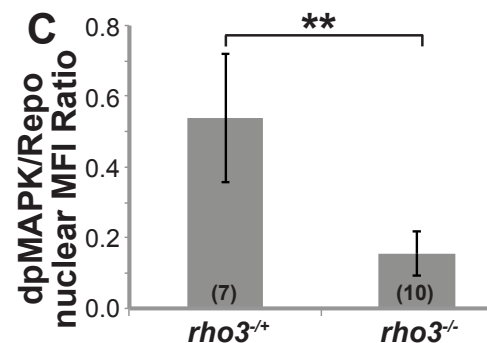
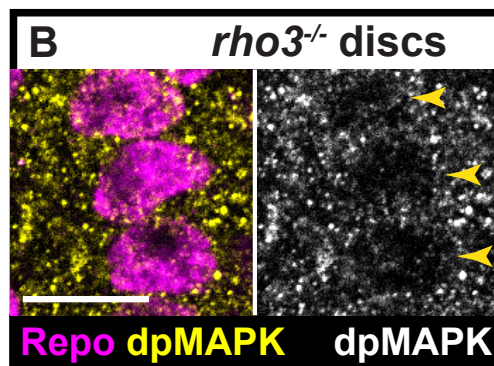
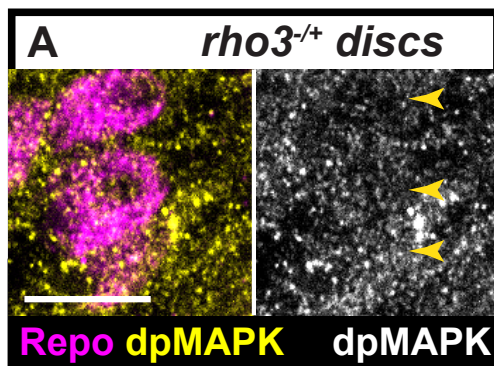


**Fig. 1**

**Photoreceptors do not communicate directly with lamina precursors through EGF.**

(A) Schematic of lamina development in the optic lobes, which is coupled to photoreceptor development in the eye disc. Hh from photoreceptors drives lamina precursor (purple) birth and assembly into columns. Photoreceptor-EGF is required for precursor differentiation into neurons (yellow). Columns consist of 6-7 precursors, which differentiate in an invariant spatiotemporal pattern (yellow). (B) A horizontal view of an early pupal (P10-15hrs APF) eye disc and optic lobe showing photoreceptor axons marked by HRP (cyan). In the optic lobe, lamina precursors express Dac (magenta) and differentiated photoreceptors and neurons express Elav (yellow). Lamina cell bodies (magenta) are organized into columns that associate with photoreceptor axons. Wrapping glia, marked by membrane-targeted GFP (white) driven by a wrapping glia-specific Gal4, extended processes through the optic stalk and into the lamina, where they encapsulate lamina cells and photoreceptors progressively (inset in B''; arrowheads mark location of photoreceptors between glial processes and lamina cells). (C) Expressing Htl<sup>DN</sup> in wrapping glia disrupted glial process infiltration into the lamina. Only cells immediately below glial processes differentiated (arrowhead in D''). (D) Lamina-specific Gal4 driving GFP showed normal lamina neuron differentiation. (E) Lamina-specific EGFR<sup>DN</sup> and P35 co-expression did not affect neuronal differentiation. (F) Lamina-specific Aop<sup>ACT</sup> and P35 co-expression led to loss of differentiated neurons (dashed bracket). (G) Lamina-specific MAPK<sup>ACT</sup> expression led to premature Elav expression in columns. (H) Quantification of (E-F) as a percentage of differentiated cells in the 6 youngest lamina columns. Asterisks indicate significance with Mann-Whitney U-test  $p < 0.01$ ; #optic lobes examined indicated in brackets. (Scale bar = 10 $\mu$ m).

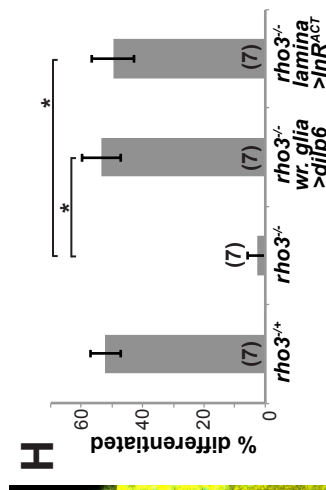
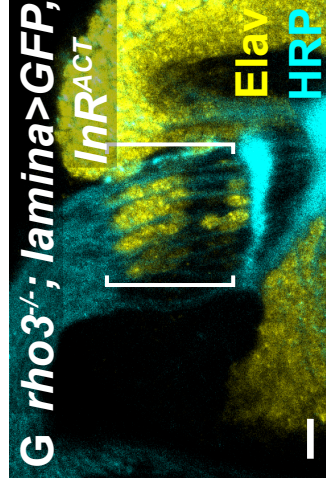
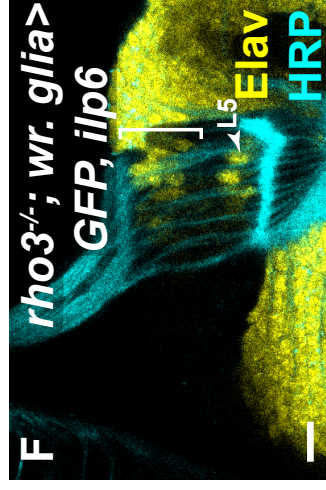
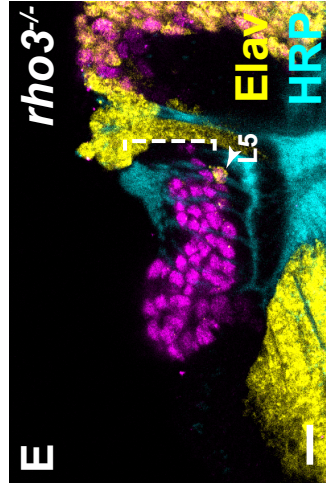
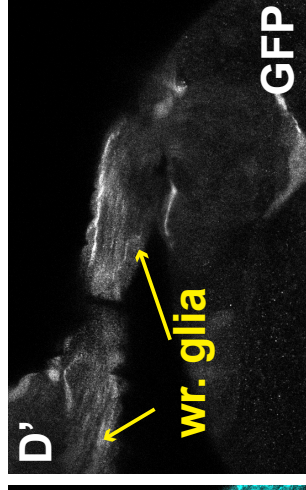
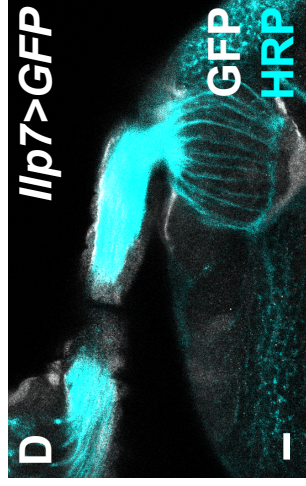
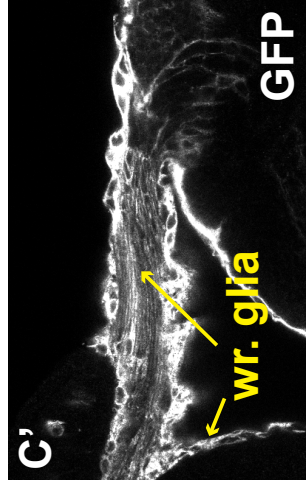
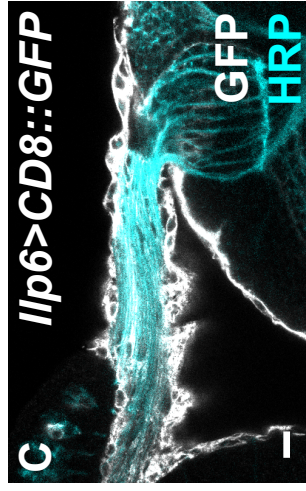
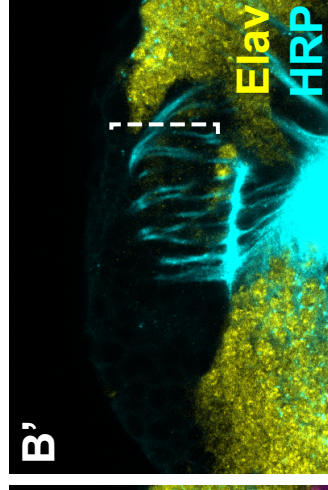
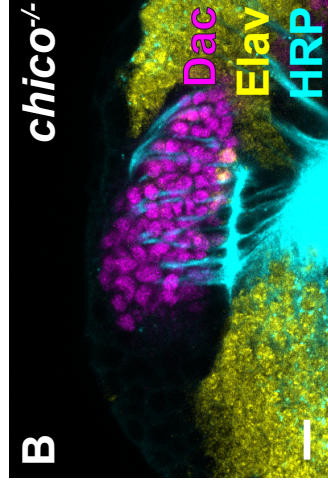
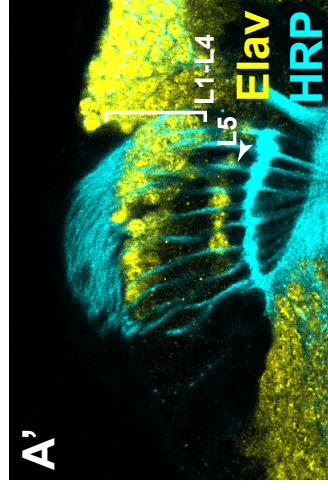
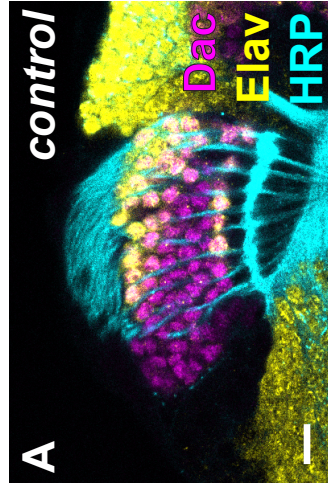




**Fig. 2****L1-L4 differentiation requires photoreceptor-induced EGFR signaling in wrapping glia**

Eye discs with wrapping glia marked by the pan-glial nuclear marker Repo (Magenta) and dpMAPK (yellow) in **(A)**  $\rho\text{ho}3^{-/+}$  and **(B)**  $\rho\text{ho}3^{-/-}$  animals, quantified in **(C)**  $p < 0.001$ ; Mann-Whitney U-test; #discs indicated in brackets. **(D-G)** Optic lobes stained for Elav (yellow), Dac (magenta), HRP (cyan) and GFP (white) **(D)** A control *wr. glia > GFP* lamina. **(E)** When wrapping glia express  $\text{EGFR}^{\text{DN}}$ , only presumptive L5s differentiated (arrow head). **(F)** In a  $\rho\text{ho}3^{-/-}$  animal, there was only a late differentiating presumptive L5 (See also Fig. S1C-G). **(G)** When wrapping glia express  $\text{EGFR}^{\text{ACT}}$  and GFP in a  $\rho\text{ho}3^{-/-}$  background, the L1-L4 front of differentiation is restored (bracket). **(H,I)** Developmentally expressed subtype-specific markers used in combination to identify neuronal subtypes (16): Sloppy paired 2 (Slp2) alone marks L2 and L3; Slp2 and Seven up (Svp) together mark L1, Brain-specific homeobox (Bsh) alone marks L4, and Slp2 and Bsh together mark L5 (dashed line indicates lamina plexus). **(H)** In a control  $\rho\text{ho}3^{-/+}$  brain and **(I)** when wrapping glia drive  $\text{EGFR}^{\text{ACT}}$  (and GFP; not shown) in a  $\rho\text{ho}3^{-/-}$  background, all cell types were recovered. **(J)** Quantification of (D-G) as a percentage of differentiated cells in the 6 youngest lamina columns. Asterisks indicate significance with Mann-Whitney U-test  $p < 0.01$ ; #optic lobes examined indicated in brackets. (Scale bar = 10  $\mu\text{m}$ ).



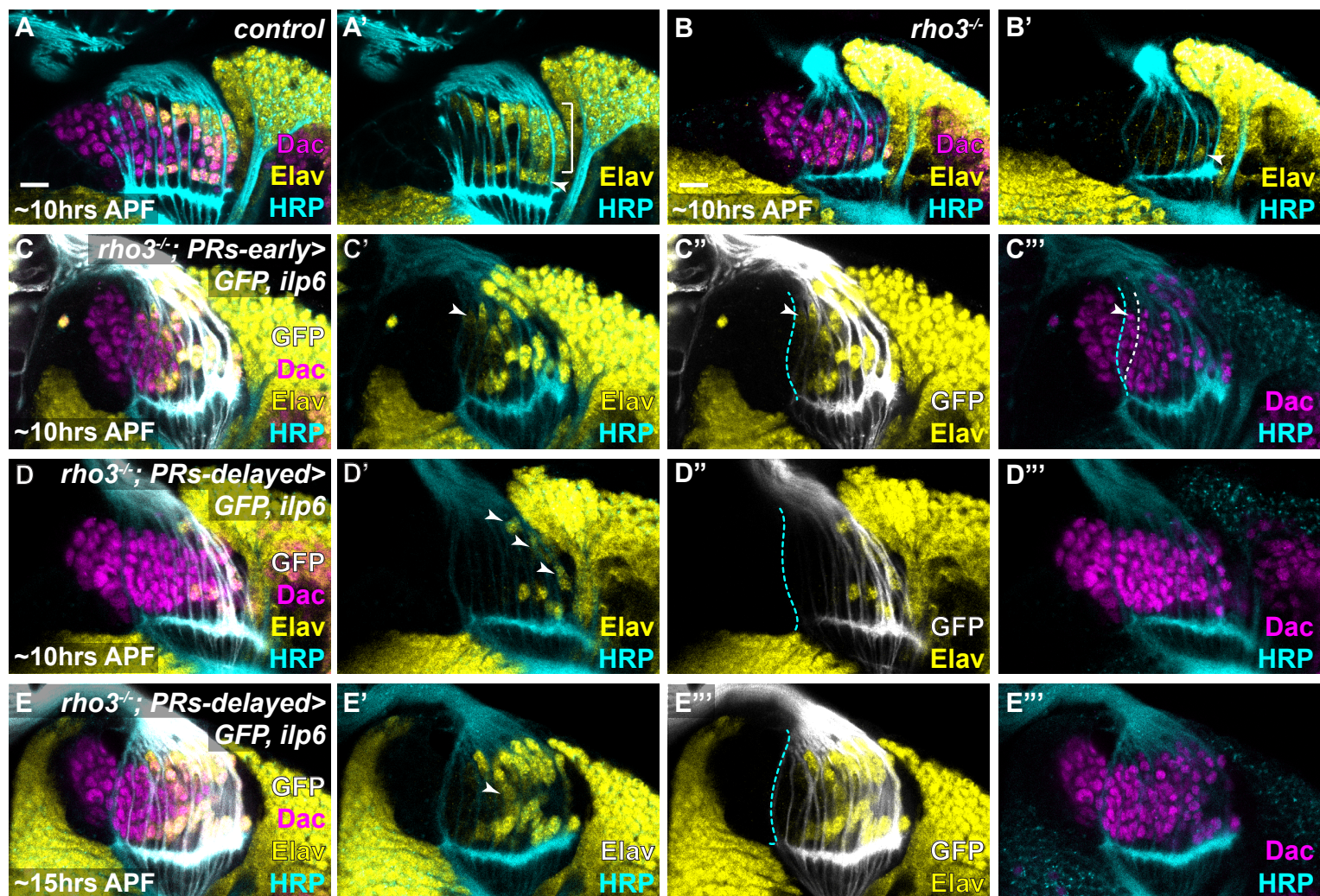


**Fig. 3**

**Wrapping glial Insulin-like peptides induce lamina neuronal differentiation**

(A) Normal lamina neuronal differentiation in a control. (B) A *chico*<sup>-/-</sup> brain lacked L1-L4 differentiation (dashed bracket). (C) Ilp6-Gal4 and (D) Ilp7-Gal4 drove expression of GFP (membrane or cytoplasmic, respectively) in wrapping glia and their extensions into the optic stalk (yellow arrows). (E) A *rho3*<sup>-/-</sup> lamina. (F) A *rho3*<sup>-/-</sup> animal with wrapping glia expressing Ilp6 showed L1-L4 differentiation (bracket). (G) A *rho3*<sup>-/-</sup> animal with the lamina expressing InR<sup>ACT</sup> showed neuronal differentiation (bracket). Elav (yellow), Dac (magenta), HRP (cyan) and GFP (white). (H) Quantification of (F,G) as a percentage of differentiated cells in the 6 youngest lamina columns. Asterisks indicate significance with Mann-Whitney U-test  $p < 0.01$ ; #optic lobes examined indicated in brackets. (Scale bar = 10 $\mu$ m).

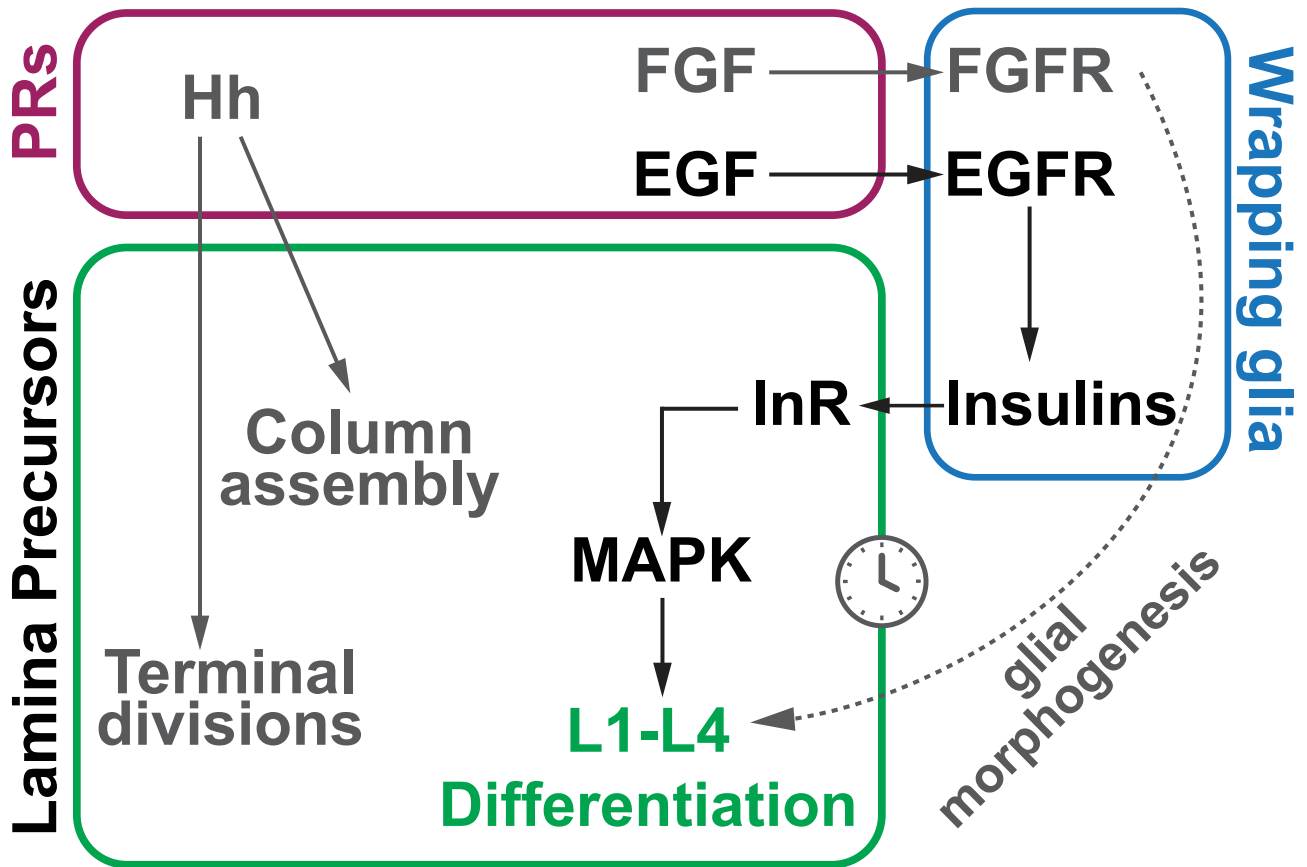




**Fig. 4**

**The signaling relay may serve to delay differentiation to ensure consistent column assembly**

(A-D) Early pupal (stages indicated) eye-optic lobe complexes stained for Elav (yellow), Dac (magenta), HRP (cyan) and (C, D) GFP (white). Cyan dashed line marks the youngest photoreceptors. (A) Control. (B) *rho3<sup>-/-</sup>*. (C) An early-onset pan-photoreceptor Gal4 driving GFP and Ilp6 in a *rho3<sup>-/-</sup>* background. Differentiation was widespread and initiated in the youngest column (arrowhead), which contained ~4 lamina precursors. (D-E) A late-onset pan-photoreceptor Gal4 driving GFP and Ilp6 in a *rho3<sup>-/-</sup>* background. (D) At ~10hrs APF, differentiation initiated only in old columns (arrowheads), but columns assembled 6-7 lamina precursors/column. (E) At ~15hrs APF, GFP and Ilp6 were expressed in all photoreceptors. Differentiation was widespread but variable as some columns contained more differentiated neurons than their older neighbors (arrowhead). (Scale bar = 10µm).





**Fig. 5****A signaling relay from photoreceptors to glia to lamina precursors instructs lamina differentiation**

Model: Photoreceptors secrete EGF and FGF, which activate EGFR and FGFR respectively in wrapping glia. EGFR activation is required for glial expression of Ilps, which activate InR and MAPK in lamina precursors leading to L1-L4 differentiation. FGFR signaling regulates glia morphogenesis and process extension into the brain (3) and therefore indirectly regulates the timing and patterning of L1-L4 differentiation.

**Supplementary content includes:**

Materials and Methods

Supplementary text

Figs. S1 to S4

Tables S1 to S2

Movies S1 to S3



## Supplementary Materials for

### **Glia relay differentiation cues to coordinate neuronal development in *Drosophila***

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Claude Desplan

\*Correspondence to [vilaiwan@nyu.edu](mailto:vilaiwan@nyu.edu)

#### **This PDF file includes:**

Materials and Methods  
Supplementary text  
Figs. S1 to S4  
Tables S1 to S2  
Captions for Movies S1 to S3

#### **Other Supplementary Materials for this manuscript includes the following:**

Movies S1 to S3

## Materials and Methods

### Immunohistochemistry

We dissected eye-optic lobe complexes from early pupae (10-15hrs After Puparium Formation or APF, unless otherwise stated; For *chico* and *InR* mutants and Imp-L2 overexpression, animals were age matched) in 1X phosphate-buffered saline (PBS), fixed in 4% formaldehyde for 20 minutes, blocked in 5% normal donkey serum and incubated in primary antibodies diluted in block overnight at 4°C. Samples were then washed in 1X PBS with 0.5% TritonX (PBSTx), incubated in secondary antibodies diluted in block, washed in PBSTx and mounted in SlowFade (Life Technologies).

We used the following primary antibodies in this study: mouse anti-Dac<sup>2-3</sup> (1:20, Developmental Studies Hybridoma Bank; DSHB), mouse anti-Repo (1:20, DSHB), rat anti-Elav (1:100, DSHB), chicken anti-GFP (1:400; EMD Millipore), mouse anti-Svp (1:50, DSHB), rabbit anti-Slp2 (1:100; Segmentation antibodies), guinea pig anti- Bsh (1:500; Chi Hon Lee), AlexaFluor405 conjugated Goat Anti-HRP (1:100; Jackson Immunochemicals), Cy3 conjugated Goat Anti-HRP (1:200; Jackson Immunochemicals), AlexaFluor647 conjugated Goat Anti-HRP (1:200; Jackson Immunochemicals). Secondary antibodies were obtained from Jackson Immunolabs or Invitrogen and used at 1:800.

### Microscopy

Images were acquired using a Leica SP5 confocal microscope. Mean Fluorescence intensity measurements and image processing were performed in Fiji. Images were compiled in Adobe Photoshop (CS5) and Illustrator (CS5).

### Fly stocks and genetics

We used the following mutant and transgenic flies in combination or recombined in this study (See Table S2 for more details; {} enclose individual genotypes, separated by commas): {yw;;}, {yw,*hsflp*<sup>122</sup>; *sp/Cyo*; *TM2/TM6B*}, {w<sup>1118</sup>;;*R94A08-Gal4*} (BL40673), {;;*rho3*<sup>PLLB</sup>, *UAS-CD8::GFP/TM6B*} (a gift from B. Shilo), {*UAS-CD8::GFP*;;}, {;;*UAS-CD8::GFP*}, {*UAS-EGFR*<sup>DN</sup>; *UAS-EGFR*<sup>DN</sup>} (BL 5364), {*UAS-λ<sup>top</sup>*;;} (a gift from E. Bach), {yw; *UAS-htl*<sup>DN</sup>; *UAS-htl*<sup>DN</sup>} (BL5366), {w<sup>1118</sup>; *UAS-dof*<sup>RNAi</sup>;} (VDRC21317), {*ey-Gal80*; *sp/Cyo*;} (BL35822), {*Gal80<sup>ts</sup>*; *TM2/TM6B*} (BL7108), {w; *UAS-Ras*<sup>V12</sup>;} (BL64196), {w<sup>1118</sup>;;*R94A08-Gal4*} (BL48073), {;;*UAS-P35*} (BL5073), {*UAS-aop*<sup>ACT</sup>;} (Kyoto Stock Center 108425), {yw;*UAS-rl<sup>sem</sup>*;} (BL59006), {yw, *hsflp122/+*; *Actin.FRT.y+.**FRT.Gal4*, *UAS-GFP*;} (a gift from E. Bach), {yw;;*UAS-s.ImpL2-HA*} (a gift from E. Hafen), {*UAS-Ilp6::FLAG*;} (a gift from P. Leopold), {w<sup>DAH</sup>; *chico*<sup>1</sup>;;}, {w<sup>DAH</sup>; *chico*<sup>1</sup>; *BAC[chicoWT-rescue]*}, {w<sup>DAH</sup>; *chico*<sup>1</sup>; *BAC[chico-PI3K binding mutant rescue]*}, {w<sup>DAH</sup>; *chico*<sup>1</sup>; *BAC[chico-Grb2 binding mutant rescue]*} (gifts from L. Partridge), {yw; *UAS-InR*<sup>R418P</sup>;} (BL8250), {*NP1079-Gal4*;;} (Kyoto Stock Center), {;;*Ilp7-Gal4*, *UAS-GFP*} (gift from Y.N. Jan), {*Ilp6*<sup>41</sup>;;} (BL30885), {*Ilp7*<sup>1</sup>;;} (BL30887), {;;*InR*<sup>E19</sup>/*TM2*} (BL9646), {;;*InR*<sup>339</sup>/*TM3*} (a gift from D. Barbosa).

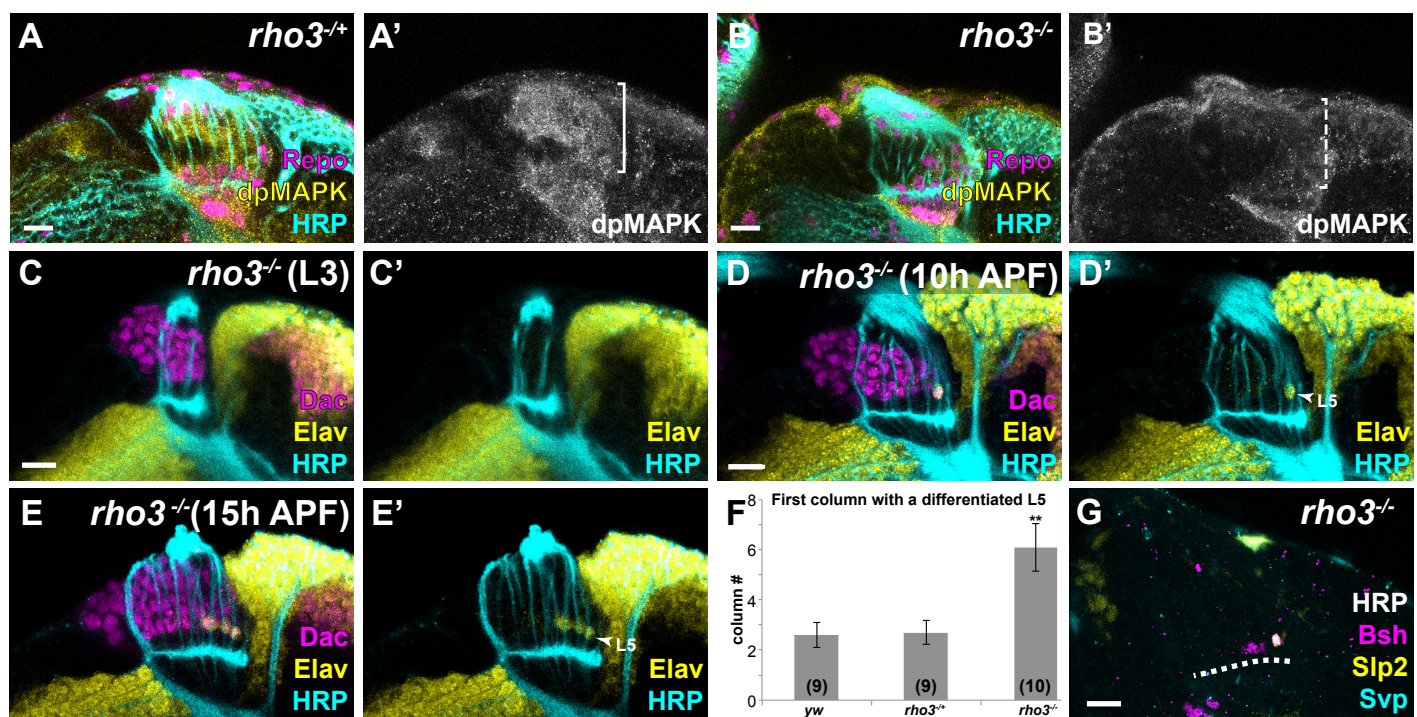
## Supplementary text

### Activating Ras/MAPK signaling in the lamina can bypass EGF from photoreceptors for neuronal differentiation.

We asked whether ectopically activating MAPK in lamina precursors could autonomously rescue lamina differentiation even in the absence of EGF from photoreceptor axons. Driving MAPK<sup>ACT</sup> in lamina precursors in a *rho3* mutant background rescued differentiation in the lamina (Fig. S2E), MAPK<sup>ACT</sup>-rescued laminas also contained differentiating L1-L4 cells (Fig. S2F). We note that the arrangement of neuronal types within columns was disrupted and we observed some neurons that expressed novel combinations of subtype-specific markers (Figs. S2F, See also Fig. S4 and Table S1). Similar results were obtained when the same rescue was performed with an activated form of Ras (Ras<sup>V12</sup>), a component upstream of MAPK (Fig. S2G). Together these data indicate that lamina neuronal differentiation depends on MAPK activation in lamina precursors downstream of a Receptor Tyrosine Kinase, distinct from EGFR. However, uniform MAPK activation in lamina precursors, lacking spatio-temporal information, was unable to generate correct neuronal fates.

### Insulin signaling through MAPK but not PI3K is required for lamina neuronal differentiation.

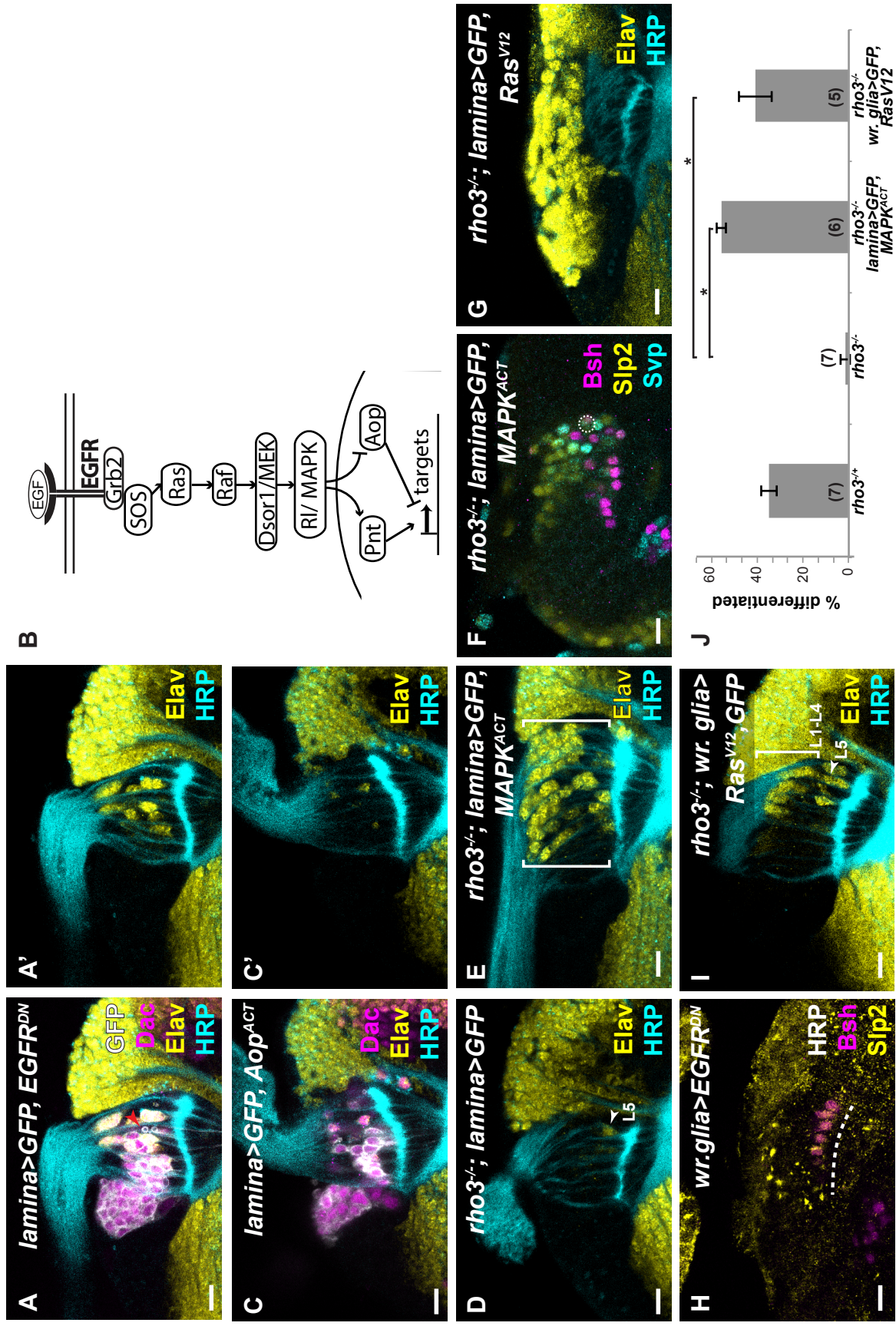
Since InR and Chico can signal through both PI3K and MAPK signaling (21, 22), we used Chico constructs that rescue both or only one or the other of the downstream signaling pathways (21) to determine which pathway or both are required for neuronal differentiation in the lamina. A wildtype Chico construct (Chico<sup>WT</sup>), where both the MAPK and PI3K branches of Insulin/Insulin-like Growth Factor signaling were restored (21), rescued the lamina differentiation defects of *chico* mutants (Fig. S3C). A Chico construct with a mutated binding site for PI3K (Chico<sup>PI3K</sup>), in which only the MAPK branch of signaling is restored (21), was also able to rescue the *chico* mutant lamina differentiation defects (Fig. S3D). In contrast, a Chico construct with a mutated Grb2 binding site (Chico<sup>Grb2</sup>), in which PI3K but not MAPK signaling is restored (29), could not rescue *chico* mutant lamina differentiation defects (Fig. S3E). These results suggest that InR signaling through MAPK but not PI3K is required for lamina neuronal differentiation, consistent with our ability to induce differentiation of lamina neurons with MAPK<sup>ACT</sup> and Ras<sup>V12</sup> expression in lamina precursors (Fig. S2E,F).



**Fig. S1**

(A) In *rho3<sup>-/+</sup>* animals, dpMAPK (yellow) was detected in the developing laminae, but (B) in *rho3<sup>-/-</sup>* animals dpMAPK levels were reduced although expression was maintained in some non-lamina cell types (Repo positive glial cell below the lamina plexus; Magenta). (C-E) *rho3<sup>-/-</sup>* eye-optic lobe complexes stained for Elav (yellow), Dac (magenta) and HRP (cyan) at different developmental time points: (C) in the third larval instar there was no lamina neuron differentiation, (D) at 10hrs APF there was a late differentiating neuron in the bottom row (arrowhead) and (E) at 15hrs APF there were a few late differentiating neurons in the bottom row (arrowhead). (F) Quantification of the delay in differentiation of lamina neurons in the bottom row in terms of the number of columns from the first column associated with PRs near the lamina furrow till an Elav expressing cell is observed (between *wildtype*, *rho3<sup>-/+</sup>* and *rho3<sup>-/-</sup>* animals; asterisks indicate Mann-Whitney U-test  $p < 0.001$ ; #optic lobes examined indicated in brackets). (G) In a *rho3<sup>-/-</sup>* brain, only a few Bsh positive cells, which start to express Slp2 (i.e. L5 neurons), were observed. (dashed line indicates position of the lamina plexus in E-F) (Scale bar = 10 $\mu$ m).

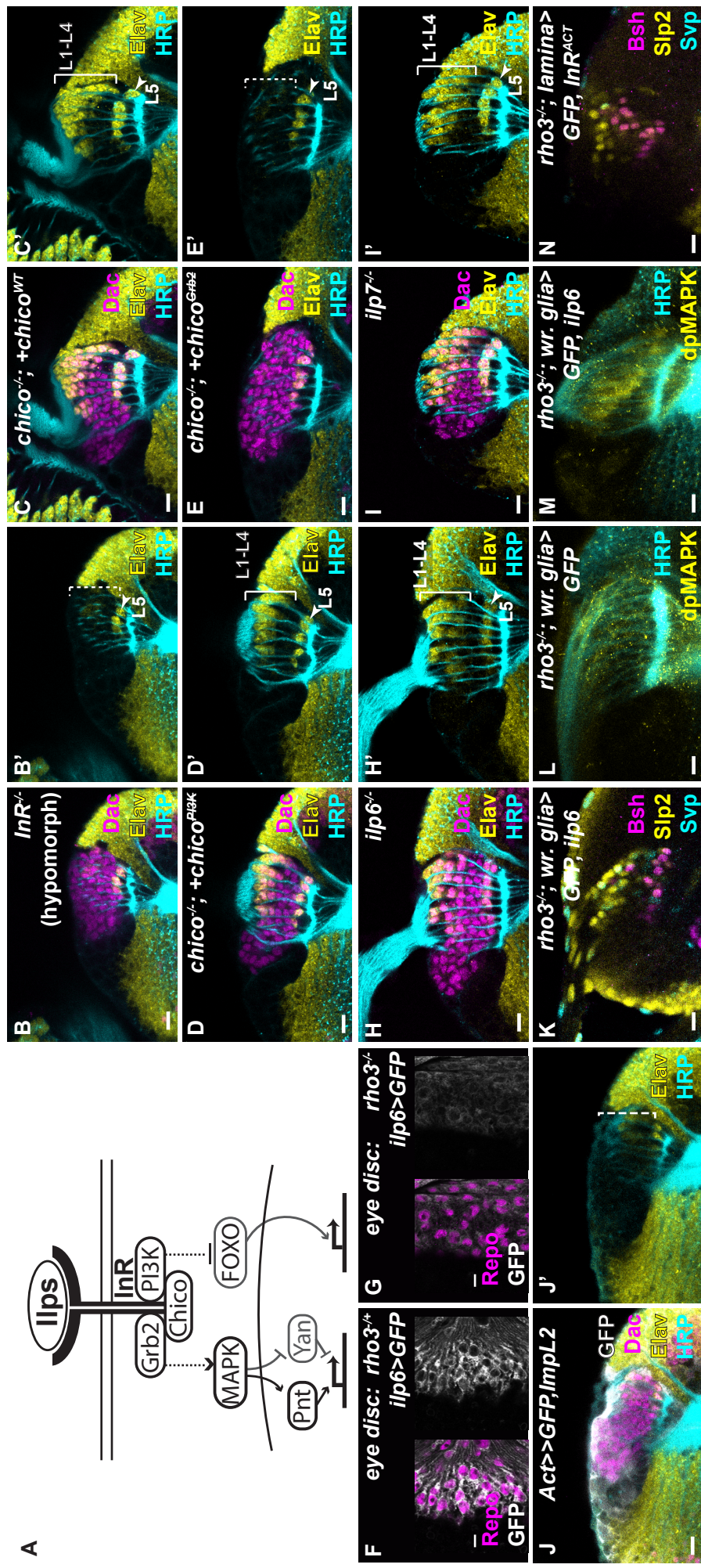






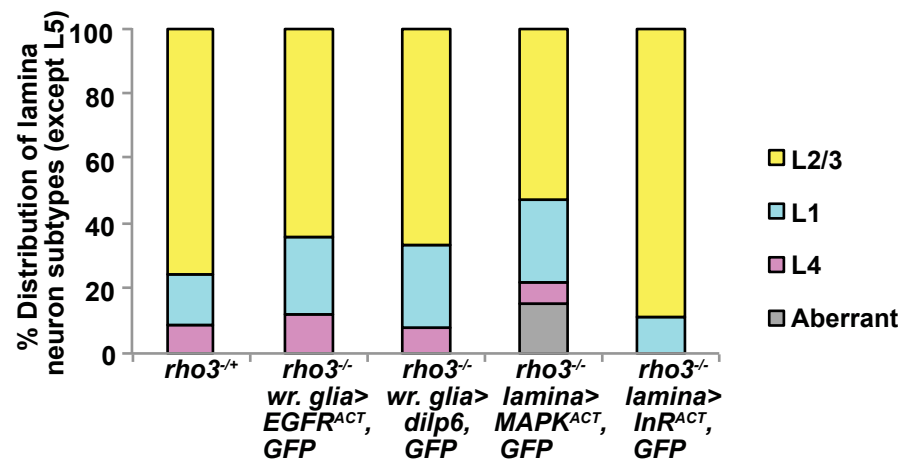
## Fig. S2

(A,C, D,G,I) eye-optic lobe complexes stained for Elav (yellow), Dac (magenta) and HRP (cyan). (A) Small fragmented cells were (arrowhead) observed together with differentiating lamina neurons when a lamina-specific Gal4 drove expression of EGFR<sup>DN</sup> and GFP. (B) Schematic of EGFR signaling pathway. (C) When Aop<sup>ACT</sup> and GFP were co-expressed in the lamina, only 2-3 cells were present in each column and lamina neuron differentiation was very sparse. (D) Only late differentiating L5s were observed in a *rho3*<sup>-/-</sup> lamina (arrowhead). (E) Lamina-specific expression of MAPK<sup>ACT</sup> in a *rho3*<sup>-/-</sup> background rescued differentiation. (F) All lamina subtypes were recovered when MAPK<sup>ACT</sup> was expressed in a *rho3*<sup>-/-</sup> background (Slp2 in yellow; Svp in cyan; Bsh in magenta; See Fig. S4 and Table S1). (G) A *rho3*<sup>-/-</sup> optic lobe expressing Ras<sup>V12</sup> and GFP (white) in the lamina. Lamina morphology was perturbed (and could not be quantified) but most cells express Elav indicating that they have differentiated. (H) In a *wrapping glia>EGFR<sup>DN</sup>* brain, only L5s marked by Slp2 and Bsh were present. (I) A *rho3*<sup>-/-</sup> animal with wrapping glia expressing Ras<sup>V12</sup> and GFP. The front of differentiation was restored in the lamina (bracket). (J) Quantification of (E,I) as a percentage of differentiated cells in the 6 youngest lamina columns. Asterisks indicate significance with Mann-Whitney U-test p<0.01; #optic lobes examined indicated in brackets. (Scale bar = 10µm).



### Fig. S3

(A) Schematic of the Insulin/Insulin-like Growth Factor signaling pathway, branching into MAPK and PI3K signaling at the Insulin Receptor Substrate, Chico. (B-G) Eye-optic lobe complexes stained for Elav (yellow), Dac (magenta), HRP (cyan). (B) A trans-allelic *InR*<sup>-/-</sup> hypomorph showed reduced L1-L4 differentiation (dashed bracket) with some presumptive L5s still present (arrowhead). (C) A *chico*<sup>-/-</sup> mutant rescued by a wildtype Chico construct (+*chico*<sup>WT</sup>) showed normal neuronal differentiation in the lamina. (D) A *chico*<sup>-/-</sup> mutant rescued by a Chico construct with a mutated binding site for PI3K (+*chico*<sup>PI3K</sup>) showed normal neuronal differentiation in the lamina. (E) A *chico*<sup>-/-</sup> mutant rescued by a Chico construct with a mutated binding site for Grb2 (+*chico*<sup>Grb2</sup>), lacks L1-L4 differentiation (dashed line). Lamina neuron differentiation appeared normal in (F,G) Eye imaginal discs showing *ilp6>2xCD8::GFP* (white) and the pan-glial nuclear marker, Repo (magenta) in a (F) *rho3*<sup>+/-</sup> control and (G) a *rho3*<sup>-/-</sup> mutant animal. *Ilp>2xCD8::GFP* expression was reduced in the *rho3*<sup>-/-</sup> mutant background. (H) *Ilp6*<sup>-/-</sup> and (I) *Ilp7*<sup>-/-</sup> null mutants. (J) A large Actin flip-out clone, marked by GFP, expressing the secreted insulin antagonist, ImpL2, showed impaired lamina differentiation (dashed bracket). (K) All neuronal subtypes were recovered when wrapping glia express *Ilp6* in *rho3*<sup>-/-</sup>, though their columnar positions are mildly disorganized, especially for L1s (Slp2 and Svp) and L4s (Bsh only) (Slp2 in yellow; Svp in cyan; Bsh in magenta; See Fig. S4 and Table S1). (L) dpMAPK expression (yellow) was low in the lamina in *rho3*<sup>-/-</sup> brain with *wrapping glia> GFP* control. (M) dpMAPK was expressed in the lamina in a *rho3*<sup>-/-</sup> eye-optic lobe complex when wrapping glia express *Ilp6* and GFP. (N) A *rho3*<sup>-/-</sup> eye-optic lobe complex rescued by lamina-specific expression of *InR*<sup>ACT</sup> stained for lamina neuron subtype-specific markers. L4s (Bsh only) are not recovered and L1s are rare (Slp2 and Svp) (See Fig. S4 and Table S1). (Scale bar = 10µm).



**Fig. S4**

Distributions of L1, L2/3, L4 and aberrant cell fates as a percentage of all subtypes (excluding L5s) in early pupal laminae (~10hrs After Puparium Formation) for:  $\{rho3^{-/+}\}$  (119 cells from 5 optic lobes),  $\{rho3^{-/-} \text{ with } wr. \text{ glia} > EGFR^{ACT}\}$  (109 cells from 5 optic lobes),  $\{rho3^{-/-} \text{ with } wr. \text{ glia} > dilp6\}$  (105 cells from 5 optic lobes),  $\{rho3^{-/-} \text{ with } lamina > MAPK^{ACT}\}$  (135 cells from 3 optic lobes) and  $\{rho3^{-/-} \text{ with } lamina > InR^{ACT}\}$  (133 cells from 3 optic lobes). See also Table S1.

**Table S1.**

Summary of lamina neuron subtypes recovered in indicated genotypes (See also Fig. S4).

<b>Genotype</b>	<b>Triangular front of differentiation</b>	<b>L1 (Svp+ Slp2)</b>	<b>L2/L3 (Slp2)</b>	<b>L4 (Bsh)</b>	<b>L5 (Bsh+ Slp2)</b>	<b>Other</b>	<b>Sub-type order</b>
<i>rho3<sup>-/+</sup></i>	Yes	Yes	Yes	Yes	Yes	No	Normal (L2/3, L1, L4, L5)
<i>rho3<sup>-/-</sup></i>	No	No	No	No	Yes (late)	No	-
<i>Wrapping glia&gt;GFP, EGFR<sup>DN</sup></i>	No	No	No	No	Yes	No	-
<i>rho3<sup>-/-</sup> Wrapping glia&gt;2xGFP, EGFR<sup>ACT</sup></i>	Yes	Yes	Yes	Yes	Yes	No	Mild disruption (Svp+ cells out of order)
<i>rho3<sup>-/-</sup> Wrapping glia&gt;2xGFP, Ilp6</i>	Yes	Yes	Yes	Yes	Yes	No	Mild disruption (Svp+ cells out of order)
<i>rho3<sup>-/-</sup> Lamina&gt;GFP, MAPK<sup>ACT</sup></i>	No	Yes	Yes	Yes	Yes	Yes (Svp+ Bsh)	Disrupted (Novel & out of order subtypes)
<i>rho3<sup>-/-</sup> Lamina&gt;GFP, InR<sup>ACT</sup></i>	No	Yes	Yes	No	Yes	No	Disrupted (out of order subtypes)

**Table S2.**

List of genotypes and conditions used for all experiments. N=#optic lobes examined or quantified.

Figure	Genotype	Conditions	N
Fig. 1B, Mov. S1	<i>;;R94A08-Gal4/UAS-CD8::GFP</i>	25°C	10/10
Fig. 1C, Mov. S2	<i>;UAS-CD8::GFP/UAS-htl<sup>DN</sup>;R94A08-Gal4/UAS-htl<sup>DN</sup></i>	29°C	6/6
Fig. 1D	<i>eyGal80/+; Gal80<sup>ts</sup>/+; R27G05-Gal4/UAS-CD8::GFP</i>	18°C for 7 days 29°C for 2 days	10/10
Fig. 1E	<i>eyGal80/+; Gal80<sup>ts</sup>/UAS-EGFR<sup>DN</sup>; R27G05-Gal4/UAS-P35</i>	18°C for 7 days 29°C for 2 days	10/10
Fig. 1F	<i>eyGal80; Gal80<sup>ts</sup>/UAS-aop<sup>ACT</sup>; R27G05-Gal4/UAS-P35</i>	18°C for 7 days 29°C for 2 days	6/6
Fig. 1G	<i>eyGal80; Gal80<sup>ts</sup>/UAS-r<sup>lsem</sup>; R27G05-Gal4/+</i>	18°C for 7 days 29°C for 2 days	6/6
Fig. 2A	<i>;;rho3<sup>PLLb</sup>, UAS-CD8::GFP/+</i>	25°C	7/7
Fig. 2B	<i>;;rho3<sup>PLLb</sup>, UAS-CD8::GFP</i>	25°C	7/7
Fig. 2D	<i>;;R94A08-Gal4/UAS-CD8::GFP</i>	25°C	9/9
Fig. 2E, Mov. S3	<i>;UAS-CD8::GFP/UAS-EGFR<sup>DN</sup>;R94A08-Gal4/+</i>	25°C	9/9
Fig. 2F	<i>;Gal80<sup>ts</sup>/+ ; rho3<sup>PLLb</sup>, R94A08-Gal4, UAS-CD8::GFP/ rho3<sup>PLLb</sup>, UAS-CD8::GFP</i>	18°C for 7 days 29°C for 2 days	9/9
Fig. 2G, I	<i>UAS-λ<sup>top</sup>/+; Gal80<sup>ts</sup>/+ ; rho3<sup>PLLb</sup>, R94A08-Gal4, UAS-CD8::GFP/ rho3<sup>PLLb</sup>, UAS-CD8::GFP</i>	18°C for 7 days 29°C for 2 days	6/6
Fig. 2H	<i>;;rho3<sup>PLLb</sup>, UAS-CD8::GFP/+</i>	25°C	7/7
Fig. 3A	<i>yw;;</i>	25 °C	
Fig. 3B	<i>w<sup>DAH</sup>; chico<sup>1</sup>;</i>	25°C	6/6
Fig. 3C	<i>NP1079-Gal4;;UAS-CD8::GFP/+</i>	25°C	5/5
Fig. 3D	<i>;;llp7-Gal4, UAS-GFP</i>	25°C	5/5
Fig. 3E	<i>;;rho3<sup>PLLb</sup>, UAS-CD8::GFP</i>	18°C for 7 days 29°C for 2 days	7/7
Fig. 3F	<i>;Gal80<sup>ts</sup>/UAS-Ilp6::FLAG; rho3<sup>PLLb</sup>, R94A08-Gal4, UAS-CD8::GFP/ rho3<sup>PLLb</sup>, UAS-CD8::GFP</i>	18°C for 7 days 29°C for 2 days	7/7
Fig. 3G	<i>eyGal80; Gal80<sup>ts</sup>/UAS-InR<sup>R418P</sup>; rho3<sup>PLLb</sup>, R27G05-Gal4/ rho3<sup>PLLb</sup>, UAS-CD8::GFP</i>	18°C for 7 days 29°C for 2 days	7/7
Fig. 4A	<i>yw;;</i>	25°C	10/10
Fig. 4B	<i>;;rho3<sup>PLLb</sup>, UAS-CD8::GFP</i>	25°C	10/10
Fig. 4C	<i>;GMR-Gal4/UAS-Ilp6::FLAG; rho3<sup>PLLb</sup>, UAS-CD8::GFP/ rho3<sup>PLLb</sup>, UAS-CD8::GFP</i>	25°C	4/4
Fig. 4D,E	<i>;GMR-Gal4/UAS-Ilp6::FLAG; rho3<sup>PLLb</sup>, UAS-CD8::GFP/ rho3<sup>PLLb</sup>, UAS-CD8::GFP</i>	25°C	4/4



Fig. S1A	<i>;;rho3<sup>PLLb</sup>, UAS-CD8::GFP/+</i>	25°C	9/9
Fig. S1B-E, G	<i>;;rho3<sup>PLLb</sup>, UAS-CD8::GFP</i>	25°C	10/10
Fig. S2A	<i>eyGal80/+; Gal80<sup>ts</sup>/UAS-EGFR<sup>DN</sup>; R27G05-Gal4/UAS-CD8::GFP</i>	18°C for 7 days 29°C for 2 days	3/3
Fig. S2C	<i>eyGal80; Gal80<sup>ts</sup>/UAS-Aop<sup>ACT</sup>; R27G05-Gal4/UAS-CD8::GFP</i>	18°C for 7 days 29°C for 2 days	3/3
Fig. S2D	<i>eyGal80; Gal80<sup>ts</sup>/+; rho3<sup>PLLb</sup>, R27G05-Gal4/ rho3<sup>PLLb</sup>, UAS-CD8::GFP</i>	18°C for 7 days 29°C for 2 days	3/3
Fig. S2E,F	<i>eyGal80; Gal80<sup>ts</sup>/UAS-r<sup>sem</sup>; rho3<sup>PLLb</sup>, R27G05-Gal4/ rho3<sup>PLLb</sup>, UAS-CD8::GFP</i>	18°C for 7 days 29°C for 2 days	6/6
Fig. S2G	<i>eyGal80; Gal80<sup>ts</sup>/UAS-Ras<sup>V12</sup>; rho3<sup>PLLb</sup>, R27G05-Gal4/ rho3<sup>PLLb</sup>, UAS-CD8::GFP</i>	18°C for 7 days 29°C for 2 days	3/3
Fig. S2H	<i>;UAS-CD8::GFP/UAS-EGFR<sup>DN</sup>;R94A08-Gal4/+</i>	25°C	See Fig.S4
Fig. S2I	<i>;Gal80<sup>ts</sup>/UAS-Ras<sup>V12</sup>; rho3<sup>PLLb</sup>, R94A08-Gal4, UAS-CD8::GFP/ rho3<sup>PLLb</sup>, UAS-CD8::GFP</i>	18°C for 7 days 29°C for 2 days	5/5
Fig. S3B	<i>;;InR<sup>ET9</sup>/InR<sup>339</sup></i>	25°C	5/5
Fig. S3C	<i>w<sup>DAH</sup>; chico<sup>1</sup>; BAC[chicoWT-rescue]</i>	25°C	6/6
Fig. S3D	<i>w<sup>DAH</sup>; chico<sup>1</sup>; BAC[chico-PI3K binding mutant]</i>	25°C	6/6
Fig. S3E	<i>w<sup>DAH</sup>; chico<sup>1</sup>; BAC[chico-Grb2 binding mutant]</i>	25°C	7/7
Fig. S3F	<i>Ilp6-Gal4;;rho3<sup>PLLb</sup>,UAS-CD8::GFP/UAS- CD8::GFP</i>	25°C	8/8 discs
Fig. S3G	<i>Ilp6-Gal4;;rho3<sup>PLLb</sup>,UAS-CD8::GFP</i>	25°C	8/8 discs
Fig. S3H	<i>Ilp6<sup>41</sup>;;</i>	25°C	4/4
Fig. S3I	<i>Ilp7<sup>1</sup>;;</i>	25°C	4/4
Fig. S3J	<i>yw, hsflp<sup>122</sup>/+; Actin.FRT.y<sup>+</sup>.FRT.Gal4, UAS- GFP/+; UAS-s.ImpL2-HA/+</i>	25°C; hs at 37°C for 2 hrs at 72hrs AEL	3/3
Fig. S3K	<i>;Gal80<sup>ts</sup>/UAS-Ilp6::FLAG; rho3<sup>PLLb</sup>, R94A08- Gal4, UAS-CD8::GFP/ rho3<sup>PLLb</sup>, UAS-CD8::GFP</i>	18°C for 7 days 29°C for 2 days	See Fig.S4
Fig. S3L	<i>;Gal80<sup>ts</sup>/+ ; rho3<sup>PLLb</sup>, R94A08-Gal4, UAS- CD8::GFP/ rho3<sup>PLLb</sup>, UAS-CD8::GFP</i>	18°C for 7 days 29°C for 2 days	4/4
Fig. S3M	<i>;Gal80<sup>ts</sup>/UAS-Ilp6::FLAG; rho3<sup>PLLb</sup>, R94A08- Gal4, UAS-CD8::GFP/ rho3<sup>PLLb</sup>, UAS-CD8::GFP</i>	18°C for 7 days 29°C for 2 days	4/4
Fig. S3N	<i>eyGal80; Gal80<sup>ts</sup>/UAS-InR<sup>R418P</sup>; rho3<sup>PLLb</sup>,R27G05-Gal4/ rho3<sup>PLLb</sup>, UAS- CD8::GFP</i>	18°C for 7 days 29°C for 2 days	See Fig.S4

**Movie S1**

Z-stack of a control eye-optic lobe complex in which a wrapping glia-specific Gal4 drives expression of membrane targeted GFP. Wrapping glia are found basally in the eye disc and wrap PRs coherently in the optic stalk and into the optic lobe. (GFP in white; Dac in magenta; HRP in cyan). (Scale bar = 10µm).

**Movie S2**

A Z-stack of an eye-optic lobe complex in which wrapping glia express membrane targeted GFP and 2 copies of Htl<sup>DN</sup>. As was previously shown, wrapping glia morphogenesis is perturbed such that large regions where PR axons are not wrapped by glia and the optic stalk appears thinner. (GFP in white; Dac in magenta; HRP in cyan). (Scale bar = 10µm).

**Movie S3**

A Z-stack of an eye-optic lobe complex in which wrapping glia express membrane targeted GFP and EGFR<sup>DN</sup>. Wrapping glial still wrap PRs coherently in the optic stalk and into the optic lobe and their overall morphology appears normal. (GFP in white; Dac in magenta; HRP in cyan). (Scale bar = 10µm).